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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/53, C12Q 1/68
G01N 33/543

A1

(11) International Publication Number: WO 94/08239

(43) International Publication Date: 14 April 1994 (14.04.94)

(21) International Application Number: PCT/GB93/02047

(22) International Filing Date: 1 October 1993 (01.10.93)

(30) Priority data:
9220773.7 2 October 1992 (02.10.92) GB

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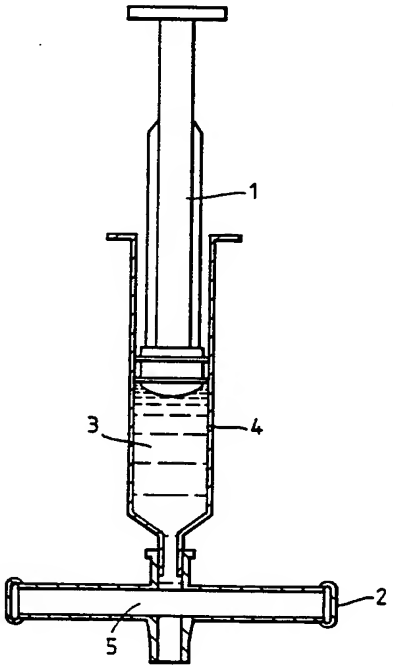
(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: DETECTION OF ANTIGENS AND NUCLEIC ACIDS

(57) Abstract

A novel application for leucocyte retention filters is provided in the form of a method and kit for isolating and/or identifying foreign antigens and/or nucleic acids associated with leucocytes in fluids derived from a human or animal body, and treating, preferably with detergent, in a manner such as to expose them for isolation and/or detection. The method and apparatus provides a simple to obtain yet rapid determination of foreign antigens and/or nucleic acids associated with the leucocytes and thus the likely presence of organisms of which these antigens are characteristic. One significant advantage of this method is that it offers the preparation of a sample outside the laboratory using a few reagents, a syringe and a filter. Using this method the inventors have assembled a pen-side test that can detect animals persistently infected with BVD virus that takes only 1.5 hours, compared to the 7 days required for a virus isolation test.



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DETECTION OF ANTIGENS AND NUCLEIC ACIDS.

The present invention relates to a method for isolating and/or identifying foreign antigens or nucleic acids associated with leucocytes in fluids derived from a human or animal body, and to kits for carrying this out. More particularly to such a method and kits as specifically adapted to be capable of use 'in the field' in so far as an electric power supply means is not essential to their operation. The method and apparatus of the present invention enable antigens to be derived from a human or animal body and identified without the need for complex laboratory bound equipment. Such method has application, inter alia, in field testing for infection of humans and animals with various infective agents having known antigenic or nucleic acid characteristics. Such testing may be carried out by a physician or veterinarian in their surgery rather than in a laboratory.

In blood processing and therapeutics arts there is a requirement to remove leucocytes from blood in order to render it suitable for transfusions or for the purpose of treating those with conditions where leucocytes are in excess, such as leukemia. There exists a large and competitive market in leucocyte 'depletion' or 'removal' filters that are capable of selective removal of leucocytes from blood for these purposes. A great number of sources of such filters will be known to those in the blood product and therapeutic arts.

The processing and therapeutic benefits of using these filters are numerous, but include, inter alia, reduction in platelet refractoriness, prevention of transmission of viruses such as cytomegalovirus, prevention of non-haemolytic febrile transfusion reactions and reduction of transfusion associated thrombocytopenia. Direct use to filter a patients blood is helpful in allowing more aggressive anticancer therapy and application to donor tissue fluids and perfusates extends organ preservation time for transplantation.

It is known that leucocytes may be analysed to determine the identity of antigens which they have immobilised in the course of their physiological function. Fenton et al (1991) described an antigen detection test for diagnosis of Border disease in sheep using leucocytes as a source of viral antigen while Mignon et al (1991) described a similar test for cattle using a monoclonal antibody for capture and signalling of presence of antigen. Such methods have focused upon analysis of leucocyte 'buffy coat' derived by methods using flash lysis and centrifugation, buffy coat separation or gradient centrifugation either alone or in combination. It will be immediately recognised that these methods require laboratory equipment that is not available in a surgery or on a farm.

Where one is assaying blood samples for organisms which produce low amounts of antigen it has proved very difficult to attain the sensitivity required to avoid false negative results. For example, diagnostics testing for Bovine Viral Diarrhoea (BVD) infection has been made difficult by the conservative nature of the virus, which is efficient in replication, and although growing in high titre, rarely produces sufficient antigen to be detected by conventional systems. Serum is the natural first choice as a sample, being readily accessible with the virus being potentially easily isolated from it. However the large variation in titre of the viraemia makes serum a potentially unreliable vector for antigen detection.

The present inventors have now provided a novel application for leucocyte retention filters in the form of a method and apparatus for isolating and/or identifying foreign antigens and/or nucleic acids associated with leucocytes in fluids derived from a human or animal body. The method and apparatus provides a simple to obtain yet rapid determination of foreign antigens and/or nucleic acids associated with the leucocytes, and thus of the likely presence of organisms of which these antigens are characteristic. One significant advantage of this method is that it offers the preparation of a sample outside the

laboratory using a few reagents, a syringe and a filter. Using this method the inventors have assembled a pen-side test that can detect animals persistently infected with BVD virus that takes only 1.5 hours, compared to the 7 days required for a virus isolation test.

The present invention provides a method for identifying foreign antigens and/or nucleic acids associated with leucocytes in fluids derived from a human or animal body, and/or isolating them in cell free form, comprising

(a) passing a sample of the fluid through a filter material having the ability to retain leucocytes thereon,

(b) treating the filter from (a) with an agent capable of acting upon leucocytes to provide foreign antigens and/or nucleic acids therefrom in elutable form,

(c) eluting antigens and/or nucleic acids from the filter,

(d) using the eluant as a source of isolated cell free foreign antigens and/or nucleic acids for performance of identification and/or further isolation steps.

The step (d) using eluant as foreign antigen and/or nucleic acid source may be carried out using standard methods well known to those skilled in the art, as will be exemplified further below.

Step (a) is conveniently carried out using any of the commercially available leucocyte retaining filter materials or filter units. These are normally comprised of many layers and trap leucocytes by both size exclusion and adsorption with those capable of retaining greater than 99.999% of leucocytes being readily available. Suitable examples are available from Bellhouse Biosciences Ltd (Abingdon-UK), Biotest (UK) Ltd (Shirley, W. Midlands (UK)), Kimal Scientific Products Ltd

(Uxbridge-UK) and Pall Biomedical Ltd (Portsmouth-UK). Some of these are capable of retaining platelets as well as leucocytes but this should not affect the method, particularly the identification step. The fluid to be filtered may be any bodily fluid or its derivative.

As absolute filtration is not necessary it is possible to use single layer filter material or filters, eg. such as those sold by Pall Process Filtration Ltd under the trademark 'Leukosorb L4'. This material is available in sheet form but is particularly conveniently available as 25mm disks in encapsulated form with a syringe-end fitting. The use of such encapsulated form allows facile use of the filter with a Leur syringe whereby use outside a laboratory becomes rapid and convenient.

Where the fluid to be filtered is whole blood an anti-clotting agent is added in an effective amount prior to subjecting the mixture to the filtration step. This agent is conveniently that such as EDTA, Na-citrate or heparin, but may be any of the other well known anti-clotting agents that will not significantly damage leucocytes.

Step (b) is carried out using any agent capable of acting upon whole leucocytes to provide the foreign antigens and/or nucleic acids in an elutable form suitable for the intended end use and is conveniently carried out after the filter has been rinsed through with a rinse liquid, eg. water, saline or buffer, eg. phosphate buffered saline. The agent is preferably a detergent and most preferably is a detergent which is capable of providing the foreign antigens associated with the leucocytes in a form whereby they are provided on the surface of micelles in the eluant.

Any detergent capable of liberating the required elutable forms from the leucocytes, and preferably acting upon the cell membranes to solubilise them, may be used but preferably non-ionic detergents will be selected. Where detergents are used which are known to

interfere with step (d) they may be removed prior to such step using adsorbents such as Bio-beads (available from Bio-Rad). A suitable range of detergents are available from suppliers such as Sigma and include the glucopyranoside range of non-ionic detergents and Nonidet P-40. Use of an optimal micelle forming amount of Nonidet takes about 1 hour contact with the filter to achieve a suitable solubilising effect. Use of preferred detergent, eg. n-octyl-β-D-glucopyranoside, achieves suitable solubilisation in about 15 minutes.

The concentration of the detergent needs to be optimised if the desired optimal micellular formation is to be achieved. For the preferred n-octyl-β-D-glucopyranoside detergent the optimal concentration is about a 2% solution, eg. in water or commercial preparation, but other suitable concentrations for this and other detergents will be derivable by those skilled in the art by simple bench experimentation using the optimised system as a measure of what can be achieved.

The step (b) is conveniently carried out by adding the detergent to the filter, eg. by passing it onto it and through its thickness, and leaving the two in contact for a period suitable for transformation of the foreign antigens and/or leucocytes to elutable form, preferably by solubilisation of the leucocytes. Using an encapsulated filter such as the Leukosorb L4 Leur syringe-end fitting filter, it is convenient to add for example 0.1 to 2 mls, preferably 0.5 mls, of a 2% n-octyl-β-D-glucopyranoside solution (eg. in water) by taking it up into a Leur syringe and using that to inject the solution onto the filter material. The syringe is conveniently that used for the taking up and applying the sample blood and rinse solution.

Step (c) is carried out after the selected time for step (b) has passed whereby the detergent solution and its contents are conveniently expelled with a rinse solution or air, preferably with air, eg. by operation of the empty syringe, application of an

air-line or by manual blowing. The expelled solution is preferably collected in a container for further processing.

Step (d) will be common to many other methods of detecting antigens or nucleic acids, or methods for further isolating these, and will be carried out after step (c) with or without an optional detergent removal step as required.

For example, for further isolation of foreign antigens, native antigens may be removed by adsorption with antibodies directed at them. Nucleic acid removal will be carried out before or after this step by methods utilising the very different properties of the materials. Alternatively, where one is trying to isolate a known antigen the eluted material may be passed down an affinity column on which are immobilised antibodies which will specifically bind the material. Techniques for isolating nucleic acids are known to those skilled in the art, eg. SDS gel chromatography against known standards.

However, particularly advantageous application of the present method is found where it is used for detection of specific foreign antigens and/or nucleic acids associated with leucocytes. In this application step (d) may be carried out a variety of methods, some of which are particularly adapted for use in the field.

For detection of specific nucleic acid the current method of choice would involve use of specifically targeted nucleic acid hybridization probes of labelled nature, these being used on their own or in combination with polymerase chain reaction primers which can be used to amplify a target sequence prior to specific probing. Such method usually involves use of radioactively labelled probes which renders it dependent upon laboratory equipment for detection of positive results. However, use of biologically labelled probes makes it possible to produce a colour forming end point on successful probing by removal of any non-hybridized material before activating the label reaction.

For detection of antigens the options for step (d) are again various, including radio immuno-assay (RIA) and enzyme linked immuno-assay (ELISA) techniques and their derivatives. More suitable for field assays however are simple slide agglutination methods utilising antibodies targeted at the antigen of interest or use of specific antibodies in immunostick 'dipstick' immobilised form.

The test kits of the present invention are those comprising components specifically associated with the method of the invention and thus comprise

(a) a filter material capable of retaining leucocytes from a fluid when that fluid is passed through it,

(b) an agent capable of acting upon filter immobilised leucocytes such that antigens and/or nucleic acids associated with them are provided in elutable form. Such combinations clearly have application in the method of the invention but not in previously noted prior art uses of leucocyte retentive filters.

Optionally provided in the kit will also be any one or more of the following components;

(c) reagents for preventing the clotting of whole blood,

(d) reagents for the specific assay of antigens and/or nucleic acids for which the kit is targeted,

(e) affinity reagents for immobilising target antigens and/or nucleic acids and optionally liberating them from their immobilised state once the fluid from which they have been separated has been removed,

(f) reagents necessary for the removal of detergent from a solution of detergent and antigen in the form of micelles.

(g) reagents necessary for the rinsing of test fluids from the filter.

For general use the reagents (a) to (g) will be those designated as preferable in the description of the method as outlined above. For field use in the preferred detection of antigens the reagents (d) are

most conveniently those of a colour forming immunoassay targeted at a specific antigen associated with an organism for which infection with the test kit is intended to detect. Most preferably the reagents (e) are those of a dipstick assay suitable for obtaining rapid detection of a target antigen in the field with a colour forming end-point.

It will be realised by those skilled in the art that virtually any foreign antigen or nucleic acid for which a suitably specific detection method, eg. binding assay, exists will be detectable by use of the present method and kits. 'Foreign' in the present context refers to material that it is not native to the human or animal whose fluids are being investigated.

The method and test kits of the invention will now be described by way of illustration only by reference to the following examples; further embodiments of the invention will occur to those skilled in the art in the light of these and the general description above.

FIGURES

Figure 1 shows a graph plotting Optical Density (OD) v Reciprocal dilution for colour assay of antigen prepared by the method of the invention (---) as compared to flash lysed and centrifuged derived antigen (___). Virus titre at neat is $10^{4.18}$ $TCID_{50}$/ml. Assay was carried out as detailed in Example 2.

Figure 2 shows a Leur syringe (1) fitted with an encapsulated Leukosorb filter (2) in a configuration for passing blood (3) from its barrel (4) onto the filter material (5).

EXAMPLE 1. Test kit for detection of Bovine Viral Diarrhoea (BVD) in cattle whole blood.

A field adapted test kit was provided consisting of:

1 x Leur syringe plus sampling needle
1 x 25mm encapsulated Leukosorb L4 filter with Leur syringe-end fitting
Phosphate Buffered Saline
2% n-octyl-β-D-glucopyranoside solution
EDTA solution or Vacutainer-EDTA K3 15% net (0.34M) 10ml tubes or Li Heparin 143 USP Unit 10 ml tubes (both tubes by Becton Dickinson).

The basic kit according to the invention was augmented by a separate set of reagents making up a dip-stick assay for BVD; such reagents optionally being provided within the same packaging as the reagents above. These reagents consisted of:
Standard solution of streptavidin-biotin-horseradish peroxidase suitable for biotin based assay,
Standard solution of tetra-methyl benzidine suitable for biotin based assay,
2 x non-competitive monoclonal antibodies (mAbs) having specificity for the p80/125 protein of BVD-prepared by conventional mAb raising techniques as ascites fluid in Balb C mice. These shown in previous work to react with high affinity to all pestiviruses tested to date. These were:
mAb WB112- on immunosticks for capture
mAb WB103- in solution in biotinylated form for signalling

The test system was first configured using polypropylene plates and then adapted to use with Nunc Maxisorb immunosticks; mAb was bound to the dipstick in carbonate buffer for 18 hours minimum. Suitable test tubes and measuring/dispensing pipettes were also provided as optional extras.

EXAMPLE 2. Method for use of test kit of Example 1.

Samples of blood were taken from cattle and treated with an amount of EDTA suitable for prevention of blood clotting. Aliquots of 5mls of the EDTA treated blood were passed through the filter using the

syringe, the syringe was used to pass a similar amount of phosphate buffered saline through the filter for rinsing purposes. The syringe was then used to pass 0.5ml of the 2% n-octyl-β-D-glucopyranoside solution onto the filter where it was left for 15 minutes before being expelled into a test tube by air. The air was provided by operation of the empty syringe.

The treated sample could be used for further isolation steps directly from the test tube but in the present example the test tube was that already containing the monoclonal antibody WB103 in biotinylated form. The dipstick from the kit was rinsed in tap water and placed into the mixture of the sample and mAb solution, as is conventional in the art, and after 30 minutes removed, again rinsed with tap water and then placed into the solution of streptavidin-biotin-horseradish peroxidase and left for a further 30 minutes. Finally the dipstick was washed again and placed in a solution of tetra-methyl benzidine wherein the colour reaction was allowed to develop for 15 minutes after which the tube was examined. Presence of a blue colour indicated the presence of the BVD characteristic antigen.

Positive and negative trials were carried out in microplates to demonstrate the reliability of the method for detecting persistent infection. A small trial of the dipstick method was carried out using EDTA blood from three persistently infected cattle and eight normal cattle. A non-dipstick ELISA carried out on the antigen produced by the filter method was carried out to assess the efficacy of the field test.

EDTA or heparin was added to blood by use of Vacutainer tubes as described in the alternatives for the kit in Example 1. Thus 10mls of blood was added to such tube direct from the sampling syringe whereby it contacts EDTA or heparin that has been coated onto the tube wall. The EDTA or heparin anti-clotting agent is provided in such an amount as to produce 0.34M EDTA or 143 USP Units per 10mls, and the

resultant treated blood was then taken up into the sampling syringe and passed onto and through the filter.

Results were obtained showing that the filter method of sample preparation successfully provided a detectable amount of antigen in blood derived from infected animals. See Figure 1 where detectability of antigen in blood derived by flash lysed and centrifugation is compared with that derived from the method of the present invention.

Further investigation into the storability of the dipstick reagent was carried out whereby it was shown that the test worked with dipsticks prepared at weekly intervals for nine weeks and kept at 4°C and room temperature in the dark. At the end of the period the performance of tests using them was assessed and found to be unimpared in all cases.

CLAIMS.

1. A method for identifying foreign antigens and/or nucleic acids associated with leucocytes in fluids derived from a human or animal body, and/or isolating them in cell free form, comprising

(a) passing a sample of the fluid through a filter material having the ability to retain leucocytes thereon,

(b) treating the filter from (a) with an agent capable of acting upon leucocytes to provide foreign antigens and/or nucleic acids therefrom in elutable form,

(c) eluting antigens and/or nucleic acids from the filter,

(d) using the eluant as a source of isolated cell free foreign antigens and/or nucleic acids for performance of identification and/or further isolation steps.

2. A method as claimed in claim 1 wherein the filter material is encapsulated in a form with a syringe-end fitting.

3. A method wherein the fluid to be filtered is whole blood and an anti-clotting agent is added to the blood in an effective amount prior to the filtration step.

4. A method as claimed in claim 3 wherein the anti-clotting agent is EDTA, Na-citrate or heparin.

5. A method as claimed in any one of the preceding claims wherein step (b) is carried out after the filter material has been rinsed through with a rinse liquid.

6. A method as claimed in claim 5 wherein the rinse liquid is water, saline or buffer.

7. A method as claimed in claim 6 wherein the buffer is phosphate buffered saline.

8. A method as claimed in any one of the preceding claims wherein the agent used for step (b) is a detergent.

9. A method as claimed in claim 8 wherein the detergent is that which is capable of providing the foreign antigens associated with the leucocytes in a form whereby they are provided on the surface of micelles in the eluant.

10. A method as claimed in claim 8 or 9 wherein the detergent is capable of solubilising the leucocytes.

11. A method as claimed in claim 8, 9 or 10 wherein the detergent is a non-ionic detergent.

12. A method as claimed in claim 11 wherein the detergent is a glucopyranoside or a Nonidet (RTM) detergent.

13. A method as claimed in any one of claims 8 to 11 wherein the detergent is removed from the eluant with adsorbent prior to carrying out step (d) or subsequent steps.

14. A method as claimed in claim 13 wherein the detergent is n-octyl-β-D-glucopyranoside.

15. A method as claimed in claim 14 wherein the detergent is a 2% solution of n-octyl-β-D-glucopyranoside.

16. A method as claimed any one of the preceding claims wherein step (c) is carried out by expelling the detergent solution from the filter material using a rinse solution and/or air.

17. A method as claimed in any one of the preceding claims wherein step (d) comprises a step of identification of specific nucleic acid by use of specifically targeted nucleic acid hybridization probes of labelled nature, on their own or in combination with polymerase chain reaction primers which can be used to amplify a target sequence prior to specific probing.

18. A method as claimed in claim 17 comprising use of biologically labelled probes in combination with reagents which produce a colour forming end point on successful probing.

19. A method as claimed in any one of claims 1 to 16 wherein step (d) comprises detection of antigens by radio immuno-assay (RIA) or enzyme linked immuno-assay (ELISA) or their derivatives.

20. A method as claimed in any one of claims 1 to 16 wherein step (d) comprises detection of antigens by slide agglutination utilising antibodies targeted at the antigen of interest or use of specific antibodies in immunostick 'dipstick' immobilised form.

21. A kit for isolating and/or detecting foreign antigens and/or nucleic acids associated with leucocytes in fluids derived from a human or animal body comprising

(a) a filter material capable of retaining leucocytes from a fluid when that fluid is passed through it,
(b) an agent capable of acting upon filter immobilised leucocytes such that antigens and/or nucleic acids associated with them are provided in elutable form.

22. A kit as claimed in claim 21 further comprising one or more of

(c) reagents for preventing the clotting of whole blood,
(d) reagents for the specific assay of antigens and/or nucleic acids for which the kit is targeted,
(e) affinity reagents for immobilising target antigens and/or nucleic acids and optionally liberating them from their immobilised state once the fluid from which they have been separated has been removed,
(f) reagents necessary for the removal of detergent from a solution of detergent and antigen in the form of micelles and,
(g) reagents necessary for the rinsing of test fluids from the filter.

23. A kit as claimed in claim 22 wherein the reagents (d) are those of a colour forming immunoassay targeted at a specific antigen associated with an organism for which infection with the test kit is intended to detect.

24. A kit as claimed in claim 23 wherein the reagents (d) are those of a dipstick assay suitable for obtaining rapid detection of a target antigen in the field with a colour formation end-point.

25. A kit as claimed in claim 24 wherein the reagents (d) are immunostick reagents with one antibody specific for the foreign antigen immobilised in immonostick form and another antibody specific for the foreign antigen labelled with a biological label.

26. A kit as claimed in claim 25 wherein the biological label is provided by biotinylation of the antibody.

27. A kit as claimed in any one of claims 21 to 26 substantially as described in Example 1.

28. A method as claimed in any one of claims 1 to 20 substantially as described in Example 2.

Fig.1.

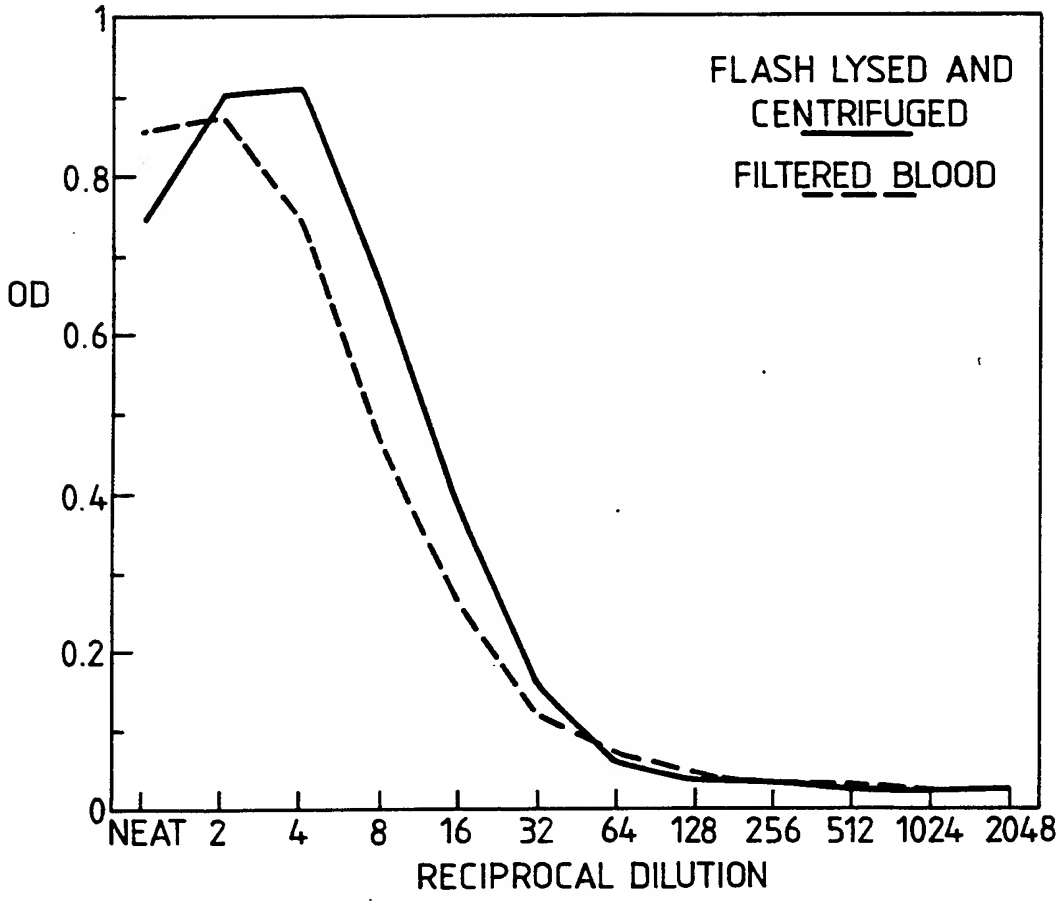
FLASH LYSED AND
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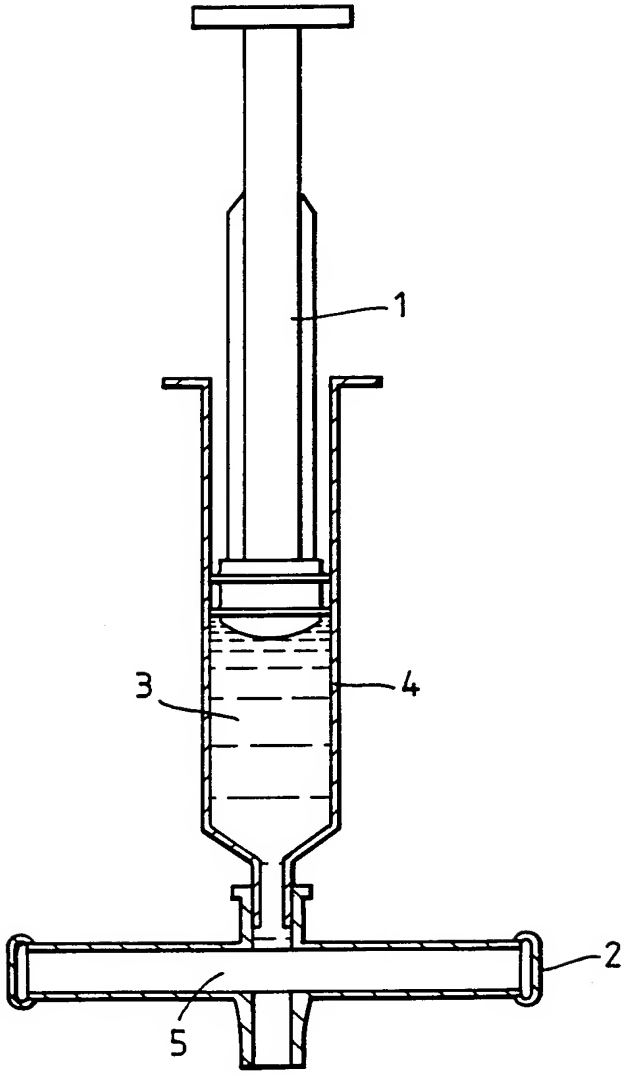
Fig. 2.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/02047

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/53 C12Q1/68 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 471 570 (LA MINA LTD.) 19 February 1992 see abstract; claim 1; figure 2	1,2
A	US,A,4 953 561 (R. A. GUIRGUIS .) 4 September 1990 see the whole document	1,2
A	WO,A,92 08981 (T CELL DIAGNOSTIC, INC.) 29 May 1992 see claims 1,4,12	8-12,19
A	US,A,4 981 685 (M. C. HEALEY) 1 January 1991 see claim 1	12,14,15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 93/02047

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		JP-A-	3131760	05-06-91
		US-A-	5042502	27-08-91
		US-A-	5139031	18-08-92
		US-A-	5224489	06-07-93
		AU-B-	644096	02-12-93
		AU-A-	6252290	21-03-91
		EP-A-	0419168	27-03-91
		JP-A-	3170060	23-07-91
		US-A-	5016644	21-05-91
		US-A-	5022411	11-06-91
		US-A-	5137031	11-08-92
WO-A-9208981	29-05-92	US-A-	5006459	09-04-91
		AU-B-	614928	19-09-91
		AU-A-	7126787	20-10-87
		CA-A-	1283045	16-04-91
		EP-A-	0261174	30-03-88
		JP-T-	1500367	09-02-89
		WO-A-	8705912	08-10-87
		WO-A-	9208980	29-05-92
		AU-A-	4416189	01-05-90
		CA-A-	2000308	06-04-90
		WO-A-	9004180	19-04-90
		AU-A-	8946591	11-06-92
		AU-A-	8956391	11-06-92
		CA-A-	2095626	08-05-92
		EP-A-	0556272	25-08-93
US-A-4981685	01-01-91	NONE		
EP-A-0464010	02-01-92	NONE		